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Influence of salinity on spermatogenesis in adult Nile tilapia (Oreochromis niloticus) testis

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ABSTRACT

Continental waters salinisation is a global threat that has grown because of climate change and human activities, but little is known about how and what biological tracts are affected. The aim of this study was to investigate the influence of different water salinities on the expression of HSP70, PCNA and caspase-3 during spermatogenesis of Nile tilapia. Adult males were submitted to four salinity treatments: (S_0) fresh water, (S_7) 7 g L^{-1}, (S_{14}) 14 g L^{-1}, and (S_{21}) 21 g L^{-1} for 1, 4, and 9 days. All specimens were in spermatogenic activity and the highest values of the gonadosomatic index (GSI) occurred in the S_0 and S_7. In the morphometric analysis, spermatocytes were the most frequent germ cell detected in all treatments (>50%) and spermatids achieved about 20% of the testicular proportion, with few variations among treatments. Spermatozoa were significantly reduced only in S14 compared to S7. Leydig cells were significantly increased in S_{14} when compared to S7 but plasma concentrations of 11-KT showed no significant difference among treatments. ELISA assay showed higher testicular expression of HSP70 at 1 day in all groups, followed by a significant decrease at days 4 and 9 in S_{14} and S_{21}. The expression of PCNA was significantly lower while the activity of caspase-3 was higher in S_{14} and S_{21} when compared to S_0 and S_7. These results indicate that higher salinities in S_{14} and S_{21} interfere with the relationship between testicular HSP70, PCNA, and caspase-3, but with few effects over spermatogenesis dynamics of Nile tilapia.

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1. Introduction

The natural salinity of rivers is a complex and dynamic process influenced by climate change, geology, distance from the sea, topography, and vegetation [1]. In addition to natural factors, human activity such as agriculture, industrial processes, and extraction of natural resources also cause an increase of salts in rivers and streams. Anthropogenic salinisation is a global and growing threat that can cause severe biodiversity loss and compromise freshwater ecosystems [1]. It occurs on all continents, but it is especially significant in the arid and semi-arid regions of Central America, South America, North America, Asia, and Australia [2]. The increase of salinity in rivers and streams may interfere with the osmotic balance, thus affecting biological functions and reducing animal performance [3,4].

The Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) (Perciformes: Cichlidae), is one of the most important freshwater fish in the world due to its excellent meat quality, rapid growth, adaptation to confinement, omnivorous habits and easy acceptance of feeding from post-larvae stages to adulthood [5,6]. Precocious sexual maturity and the ability to reproduce throughout the year at 24 °C and above [7] make the species an interesting experimental model for reproductive biology studies. Males develop rapidly, are larger than females and, therefore, have greater commercial importance [8]. Furthermore, the cystic arrangement of spermatogenesis allows a reliable morphometric analysis of the germ

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cells under different experimental conditions [9–11].

Pituitary gonadotropins, growth factors, and sex steroids regulate gonadal maturation, and 11-ketotestosterone (11-KT) is the major androgen of fish that acts on spermatogenesis progression [12]. Functionally, spermatogenesis has been divided into three distinct phases: proliferative or spermatogonial, meiotic or spermatocitary and spermiogenic [12,13], with sperm production being guaranteed by a balance between proliferation and apoptosis of germ cells [14,15]. Apoptosis requires a specialised cellular machinery involving a family of proteases called caspases, with an apoptosis-effector molecule, caspase-3, being used as a biomarker of cell death under different experimental conditions [16]. Proliferating cell nuclear antigen (PCNA) plays a role in DNA replication and repair and is widely used to study gametogenesis and embryogenesis in vertebrates [17,18].

Several environmental and physiological factors induce cells to rapidly synthesise a group of proteins called heat shock proteins (HSPs), which regulate cell metabolism, growth, differentiation, and apoptosis [19]. HSPs are expressed in all organisms and cell types and have an important role as a molecular chaperone, assisting folding, unfolding, transport and assembly of multiprotein complexes [20]. Amongst HSPs, HSP70 is constitutively expressed in different phases of spermatogenesis and is essential for testicular development [21,22]. In mice, a HSP70-2 deficiency results in the blocking of spermatogenesis in the meiosis prophase I and leads to infertility [23]. In fish, greater expression of HSP70 has been related to environmental stress, such as exposure to heavy metals and to endocrine disrupters [24]. However, the influence of salinity on HSP70 expression in fish testes has not yet been evaluated.

Despite the high environmental and economic cost, detailed studies that assess the effects of water salinity on organisms, especially fish, are scarce. Therefore, the aim of the present study was to analyse the spermatogenesis of *O. niloticus* under different water salinities through morphometric analysis of germ cells, as well as testicular levels of HSP70, PCNA, and caspase-3, relating the data obtained to the plasma concentrations of the androgen 11-ketotestosterone.

2. Material and methods

2.1. Fish sampling

The experiment was carried out in August 2013 at the Aquaculture Laboratory of the Veterinary School of the Federal University of Minas Gerais (19°52′16″S, 43° 58′14″W) in Belo Horizonte, state of Minas Gerais, Brazil. All procedures were approved by the Committee on Ethics and Animal Use (CEUA, protocol 396/2012) of the Federal University of Minas Gerais (UFMG), Brazil. Adult males of Nile tilapia *O. niloticus* with 14.19 ± 0.22 cm total length (TL) and 58.94 ± 2.64 g body weight (BW) were randomly allocated in eight 150 L tanks. The fish, 12 per tank, were submitted to four salinity treatments, in duplicates (N = 24 fishes per treatment): (S_0) control group in freshwater, (S_7) 7 g L^{-1}, (S_{14}) 14 g L^{-1}; and (S_{21}) 21 g L^{-1}. To salinize the water, we used non-iodized salt (Marisal LTDA - ingredients: Sodium chloride). To acclimation, the salt was added in three steps, reaching the highest concentration after 8 h. During the experiment, the tanks were kept with continuous aeration and photoperiod 12 L: 12 D. Fish were fed with commercial diet (32% crude protein) *ad libitum* twice daily at an amount corresponding to 2% of the biomass, and the remainder was removed after 30 min. To prevent the accumulation of ammonia, 2/3 of the water was siphoned off daily and replaced with water at the same temperature and salinity that had been previously prepared. The water quality parameters were monitored daily using a YSI multiparameter probe (Model 6920 V2), and the following values (mean ± SD) were recorded: temperature = 27.18 ± 1.07 °C; dissolved oxygen = 5.27 ± 1.07 mg L^{-1}; pH = 6.36 ± 0.16; conductivity (mS/cm) = 0.18 ± 0.05 (S_0), 12.27 ± 0.35 (S_7), 23.08 ± 0.69 (S_{14}), and 32.95 ± 1.02 (S_{21}); salinity (g L^{-1}) = 0.09 ± 0.01 (S_0), 7.10 ± 0.06 (S_7), 14.06 ± 0.01 (S_{14}), and 21.03 ± 0.02 (S_{21}).

Fish sampling (N = 8 per time) was carried out at 1, 4, and 9 days after the start of the treatment. Fish were killed by immersing them in a solution of eugenol at 285 mg L^{-1} and all the experimental procedures were carried out in accordance with the ethical principles established by the Brazilian College of Animal Experimentation (COBEA). For each specimen, gonad weight (GW) was measured to calculate the gonadosomatic index (GSI = 100 GW/BW) and the Fulton condition factor (K = 100 BW/TL^3). Blood plasma samples were obtained by cardiac puncture for hormonal dosage for ELISA assay. To test the efficiency of osmoregulation, concentration of Na^+ was recorded in testis homogenates (7 samples per treatment) using an automatic ion selective electrode (ISE), and the following values (mEq/L, mean ± SE) were obtained: 24.4 ± 1.6 (S_0), 24.6 ± 3.1 (S_7), 25.4 ± 2.1 (S_{14}) and 25.7 ± 1.9 (S_{21}), without significant difference among treatments (F = 0.07, p = 0.97, One-way ANOVA and Tukey's post-test).

2.2. Histology and morphometry of spermatogenesis

Testis samples were fixed in Bouin's fluid for 12 h, preserved in 70% alcohol, embedded in paraffin, sectioned at 5 μm thickness, stained with haematoxylin-eosin and Gomori's trichrome. The diameter of the seminiferous tubules (ST) was obtained considering the average of the largest and smallest diameters, which were measured in 20 STs randomly selected per slide at 100X magnification, totalizing 120 STs measured per treatment. The measures were made using the AxioVision image analysis system coupled to an Axioplan Zeiss microscope. The proportion (%) of germ cells was obtained at 9 days of treatment, and six fish per salinity were used in the analyses. The germ cells were identified by nuclear diameter, chromatin organization and nucleolus development, following criteria previously established [11,12,25]. Type A spermatogonia, type B spermatogonia, spermatocytes, spermatids, spermatozoa, Leydig cells, and interstitial tissue were quantified for 5 random fields of each histological section at 400X magnification, making a total of 30 fields analysed per treatment. Tubular lumen and tissue retraction were considered as white spaces in the counts. A grid with 475 intersections points between lines placed on each virtual image was used for counting using ImageJ software. The structures or cells that were under each intersection were recorded.

2.3. Plasma 11-ketotestosterone (11-KT)

The plasma concentrations of 11-KT were determined using enzyme linked immunosorbent assay (ELISA). For this, blood samples of 8 fishes of each salinity were obtained after 9 days of salinity treatment. All samples were prepared individually. These were then centrifuged for 3 min at 704 g and the serum aliquots were stored at −80 °C until steroid assay was performed. Concentrations of 11-KT were determined using the commercial ELISA kit for 11-ketotestosterone (Biosense Laboratories, Norway). The assay protocol was achieved according to the procedures recommended by the manufacturer. Assay was performed in 96-well plates in duplicates and the absorbance was measured at 405 nm using a BioTek microplate reader (BioTek Instruments, USA). The values of absorbance were then analysed by using Gen5 software (BioTek Instruments, USA) coupled to spectrophotometer. The sensitivity of the assay is 1.3 pg/ml. The standard curve was prepared by serial dilution of 11-ketotestosterone EIA Standard (Fig. S1A, Supplementary Material) and the androgen concentration was

determined for each fish.

2.4. ELISA assay for HSP70 and PCNA

Testicular samples (N = 8 per treatment) previously stored at −80 °C were ground and homogenised in a cell lysis buffer (50 mM Tris-HCl pH 8.0 with 0.02% aprotinin and 1 mM phenylmethylsulfonyl fluoride) at a ratio of 1:2 (tissue weight: buffer volume) using a homogeniser (TE-099, Tecnal, Brazil). All samples were prepared individually. The extracts were sonicated (Centrifuge 5804 R Eppendorf), centrifuged at 15,000 g for 1 h at 4 °C, and the supernatant was stored at −80 °C until analysis. Total soluble protein in each sample was determined by the Bradford method using bovine serum albumin (BSA) as standard [26]. Then, samples (100 μg/ml) were incubated for 1 h in a 96-well microplate (Nunc, Denmark) blocked with 2% BSA for 45 min and incubated again at 37 °C for 1 h with mouse monoclonal primary antibody (*anti*-HSP70 clone BRM-22 or *anti*-PCNA clone PC10, Sigma-Aldrich, USA) at a 1:500 dilution. After washing with PBS-Tween 0.05%, the plates were incubated with anti-mouse IgG secondary antibody (1: 1000, Sigma-Aldrich, USA) conjugated with peroxidase for 2 h at 37 °C. After the washings, the reaction was revealed with 200 μl of 0.04% *o*-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, USA) solution in a 0.05M phosphate-citrate buffer with 0.0025% hydrogen peroxide. The reaction was stopped by adding 50 μl of 5% H_2SO_4 in each well and the absorbance was measured at 492 nm using a microplate reader (Biotek Instruments, USA). For validation of the ELISA assays, standard curves of testicular homogenates were prepared for each protein (Fig. S1 B and C, Supplementary Material).

2.5. Colorimetric assay for caspase-3

The enzymatic activity of caspase-3 was evaluated using the caspase-3 colorimetric assay (R & D Systems, Wiesbaden-Nordenstadt, Germany). The testicular samples (N = 8 per treatment) collected at 9 days of treatment were weighed and sonicated in a cell lysis buffer with protease inhibitors. All samples were prepared individually. The homogenates were centrifuged at 15,000 g for 1 h at 4 °C and the supernatant obtained was incubated with the caspase-3 substrate (DEVD-pNA) for 2 h at 37 °C. The absorbance of each sample was recorded at 405 nm and the caspase-3 activity level was directly proportional to the colour of the reaction.

2.6. Immunolocalisation of PCNA and caspase-3

Testicular sections with 5 μm thickness were submitted to immunohistochemical reaction for the detection of PCNA (mouse monoclonal antibody clone PC-10, Sigma-Aldrich, USA) and caspase-3 (rabbit polyclonal active antibody C-8487, Sigma-Aldrich, USA). For antigen recovery, sections were boiled in 10 mM sodium citrate buffer pH 6.0 during 25 min in a microwave. Blocking of non-specific binding was done with 2% BSA in phosphate-buffered saline (PBS) for 1 h. In sequence, the primary antibody (1:100) was applied to the sections and remained overnight at 4 °C. For immunofluorescence detection, the secondary antibodies (1:500) anti-mouse IgG conjugated with Alexa Fluor 568 (Invitrogen, Molecular Probes) and anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen, Molecular Probes) were used. Nuclear DNA labelling was performed by 4,6-diamidino-2-phenyl-indole (DAPI; 1:500). For negative control, the primary antibody was omitted. The sections were analysed using a fluorescence microscope (Nikon Eclipse Ti) from the Image Processing Acquisition Center of the Federal University of Minas Gerais (CAPI-UFMG).

2.7. Statistical analysis

Data were analysed using BioEstat 5.3 software, expressed as mean ± standard error and considered significant with $p < 0.05$. After the Lilliefors normality test, one-way ANOVA and Tukey's post-test were used to compare GSI, 11-KT and PCNA values between salinity treatments. Kruskal-Wallis one way analysis of variance and Dunn post-test were used to compare the non-parametric variables: K, diameter of seminiferous tubules, proportion (%) of germ cell, as well as the testicular levels of HSP70, PCNA, and caspase-3.

3. Results

3.1. Testicular development and spermatogenesis

There was no significant variation in the GSI and K between collection days (1, 4 and 9 days) for the four treatments. In order to better express the differences between salinities, we have grouped the data of the collection days. Hence, GSI values were significantly higher in fish from S_0 (0.59 ± 0.09) than in S_{14} and S_{21} (0.33 ± 0.04 and 0.33 ± 0.06) ($F = 3.82$, $p = 0.013$), and there was no significant difference between S_0 and S_7 (Fig. 1A). The Fulton (K) condition factor did not show significant variations between treatments ($H = 4.61$, $p = 0.20$) (Fig. 1B). The seminiferous tubule diameters were significantly larger in fish from S_0 (109.8 ± 1.4 μm) ($H = 30.06$, $p < 0.0001$), and no significant difference was achieved among S_7, S_{14} and S_{21} (Fig. 1D).

Histological analysis (Fig. 2A–E) showed that all specimens were in spermatogenic activity, with testes in ripening maturity stage at all salinities (Fig. 2A–E). Spermatocyte cysts were abundant in most samples while spermatids and spermatozoa were less frequent (Fig. 2B–E). In the higher salinities (S_{14} and S_{21}) were observed presence of empty spermatogenic cysts in the seminiferous tubules, enlarged interstitial tissue, hyperplasia of Leydig cells and presence of inflammatory infiltrate (Fig. 2F–H).

To analysis the effects of the salinity on the spermatogenesis dynamics, we performed a morphometric analysis of the proportion of the germ cells in fish sampled with 9 days of treatment (Fig. 1C). We found a small proportion of type A and type B spermatogonia (G_A and G_B) in all treatments, representing less than 2% of the testicular proportion. Spermatocytes (C) were the most abundant germ cells in all treatments, accounting for more than 50% of the testicular proportion. Spermatids (T) represented about 20% of the testicular proportion, with significant difference between S_0 and S_{14}, $p < 0.05$. Spermatozoa (Z) were reduced in the higher salinities, with significant difference between S_7 and S_{14} ($H = 8.82$, $p = 0.03$). The highest proportions of Leydig cells (L) were observed in S_{14} and S_{21}, with a significant difference in S_{14} when compared to S_7 ($H = 9.01$, $p = 0.03$). White spaces (W) were similar in the four treatments, $p > 0.05$, and the proportion of interstitial tissue (I) was significantly enlarged in S_{14} in relation to the other treatments ($H = 44.37$, $p < 0.0001$).

Plasma concentrations of 11-KT ranged from 92 to 135 pg/ml (Fig. 1E), with higher values detected in S14 and S21, without significant difference between treatments ($F = 1.62$, $p = 0.20$).

3.2. Testicular expression of HSP70, PCNA, and caspase-3

To investigate the effect of salinity on the protein expression of HSP70, we have performed ELISA assay. Levels of HSP70 were analysed in the three collections days, because expression of HSP70 increases briefly after a stressful condition. As expected, higher HSP70 levels occurred at day 1 in all four treatments (Fig. 3A). In fish from S_0 and S_7, HSP70 levels were similar at days 1 and 4, and

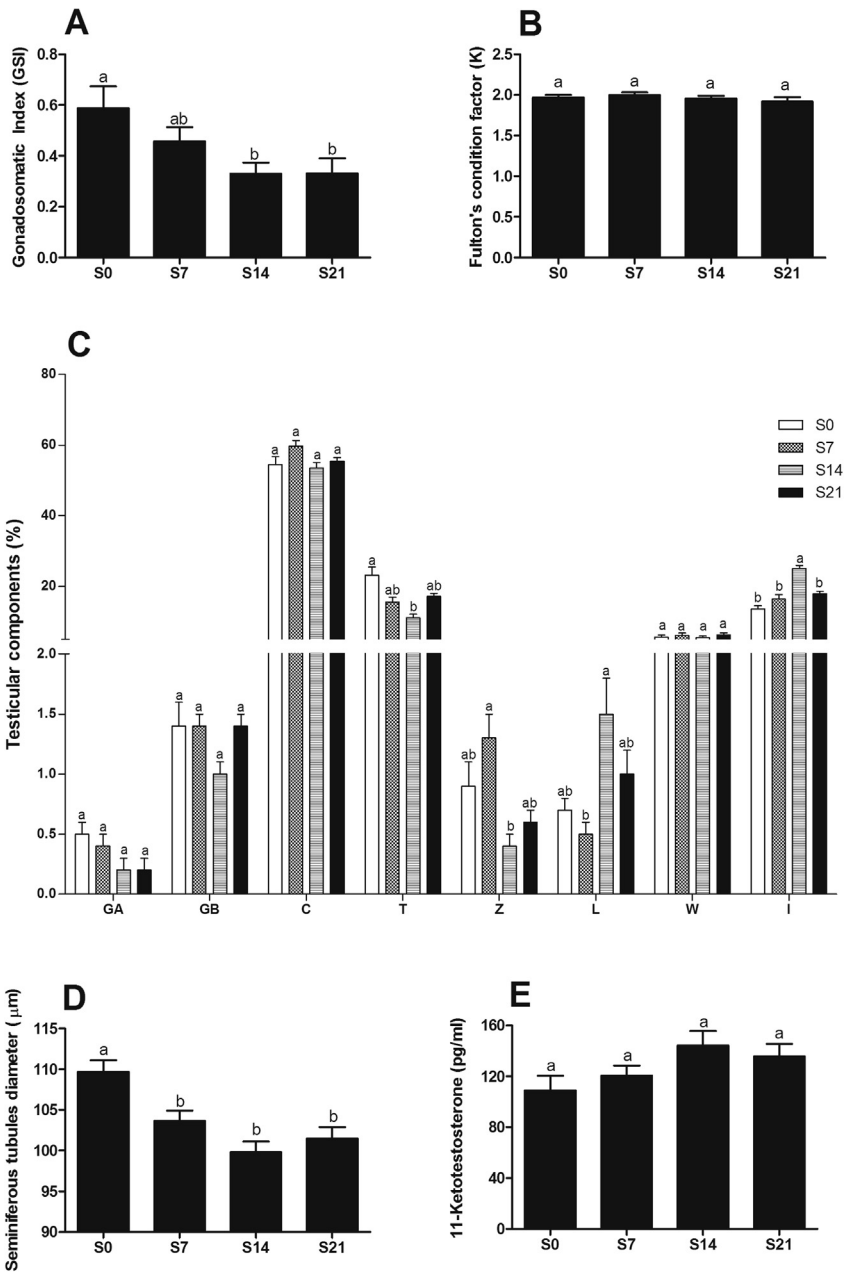

Fig. 1. (A) Gonadosomatic index, (B) Fulton condition factor, (C) Proportion (%) of testicular components, (D) Seminiferous tubules diameter, and (E) Plasma concentrations of 11-ketotestosterone. Values expressed as means ± standard error. Different letters indicate significant difference between treatments ($p < 0.05$).

then decreased at 9 days of treatment. A significant decrease of HSP70 was observed at 4 and 9 days in S_{14} and S_{21} ($H = 24.60$, $p < 0.0001$ and $H = 14.29$, $p = 0.0008$). At 9 days of treatment, PCNA levels decreased with increasing water salinity ($F = 7.36$, $p = 0.0007$), while the expression of caspase-3 increased ($H = 11.77$, $p = 0.008$) (Fig. 3B and C).

For localisation of the proteins investigated, we performed immunofluorescence. Results showed the labelling for PCNA occurring in spermatogonia (Fig. 2I). The caspase-3 immunostaining occurred in spermatids cysts and spermatozoa (Fig. 2J).

4. Discussion

Salinity affects fish communities, reduces the biomass of native species, and favours the colonisation of freshwater ecosystems by saline-tolerant species [1,27]. Nevertheless, few studies have been conducted to investigate the effects of salinity on gametogenesis and reproductive biology. The present study investigated the influence of water salinity on the fish spermatogenesis using the Nile tilapia as an experimental model. Tilapias are euryhaline species that have marine ancestors [28], so they are able to tolerate a wide range of water salinities and can grow and also reproduce in brackish waters [29]. However, salinity tolerance varies among species, and *O. niloticus* is less tolerant than other tilapia species such as *O. mossambicus, O. aureus,* and *Tilapia zilli* [29]. According, the impact of freshwater salinisation on the biota is strongly mediated by evolutionary history, as this is an important factor that determines the physiological tolerance of a species to salinity [30]. Although *O. niloticus* is a euryhaline fish, subtly higher concentrations of Na^+ were observed in testis of fishes submitted to S14 and

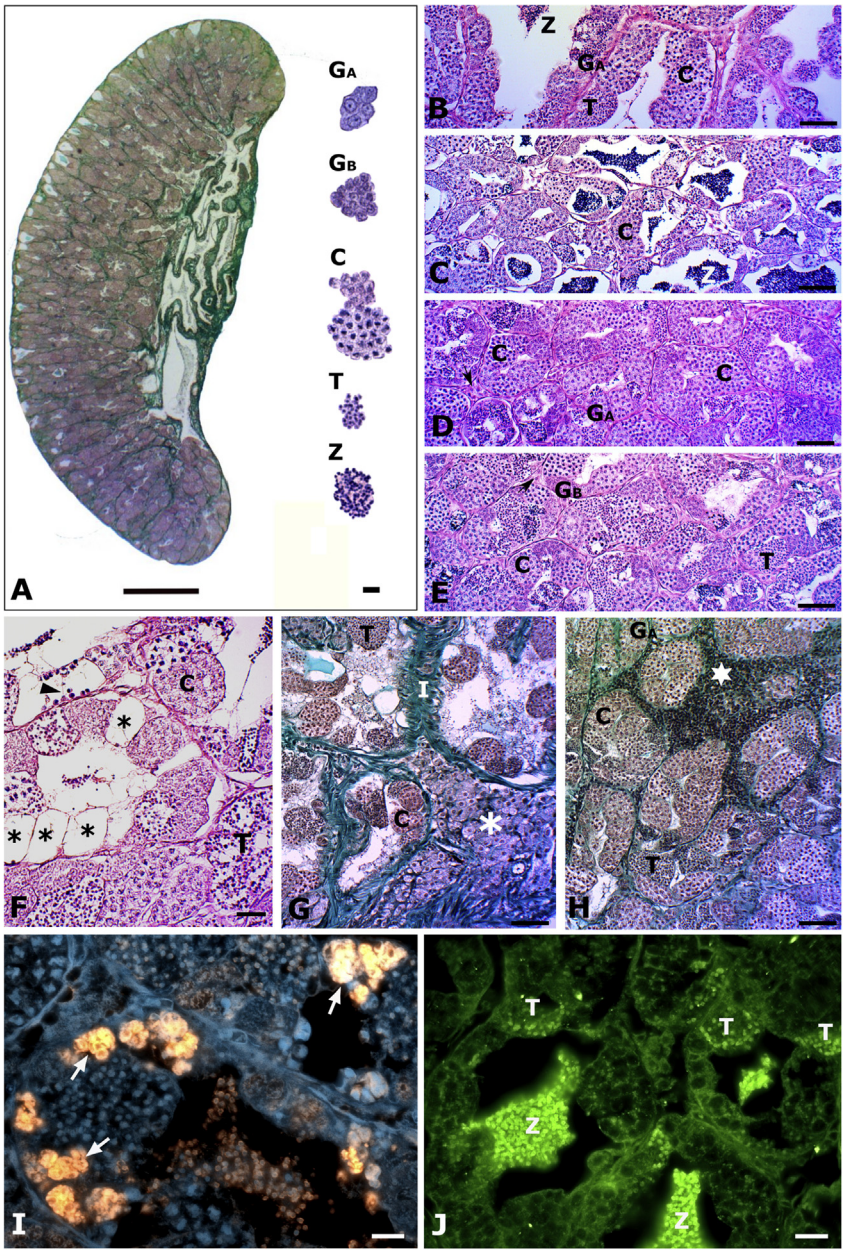

Fig. 2. Histological sections of *O. niloticus* testis stained with haematoxylin and eosin (B–F) and Gomori's trichrome (A, G, H), and immunohistochemical reaction for (I) PCNA and (J) caspase-3. (**A**) Testicular structure showing two distinct regions, and morphology of the germ cells: spermatogonia type A (G_A), spermatogonia type B (G_B), spermatocyte (C), spermatids (T), spermatozoa (Z); (B–E) Seminiferous tubules with abundant spermatocyte cysts in S_0, S_7, S_{14} and S_{21}, respectively (E); (F) Empty spermatogenic cysts (*) and granulocytes (arrowhead) in S_{21}; (G) Enlarged interstitial tissue (I) and hyperplasia of Leydig cells (asterisk) in S_{14}, (H) Inflammatory infiltrate (star) in S_{21}; (I) Spermatogonia cysts labelled for PCNA (arrow) and (J) Spermatids cysts and spermatozoa labelled for caspase-3 in S_{21}. Scale bar: 200 μm (A-testis), 8 μm (A-germ cells), 20 μm (B-H, J) and 10 μm (I).

S21 treatments, but no significant differences was detected. Also no significant differences in serum Na^+, K^+, Cl^- concentrations were found among any saline treatments in *O. mossambicus* [31]. Hence, the changes observed in the present study attributed to salinity can be widened to stenohaline species, which deal with narrow salinity variations.

In this study, we found higher GSI values in fish from S_0 and S_7, indicating a greater testicular development in fish kept in freshwater and low salinity. The increase in water salinity can lead to a higher energy expenditure, an increase in dissolved oxygen consumption and a lower food consumption [32,33], and may affect metabolism and biological functions as detected in S_{14} and S_{21} of this study. In the mosquitofish, *Gambusia holbrooki*, an increase in salinity reduces feeding ability and inter-specific aggressiveness, limiting its invasive capacity [34]. In an isotonic environment, where ionic gradients between blood and water are minimal, the energy saving is significant to increase somatic growth [35] and also for gonadal development, as found in S_0 and S_7 of this study. On the other hand, high salinities severely restrict tilapia reproduction, reducing spawning numbers and frequency, hatching success, and egg viability [29] and, in addition, reduce testicular development. In contrast, for the Atlantic salmon (*Salmo salar*), an anadromous species, seawater salinity is a strong stimulus to the onset of spermatogenesis during puberty, modulating the changes of GSI [36].

In this study, the high salinities of groups S_{14} and S_{21} did not

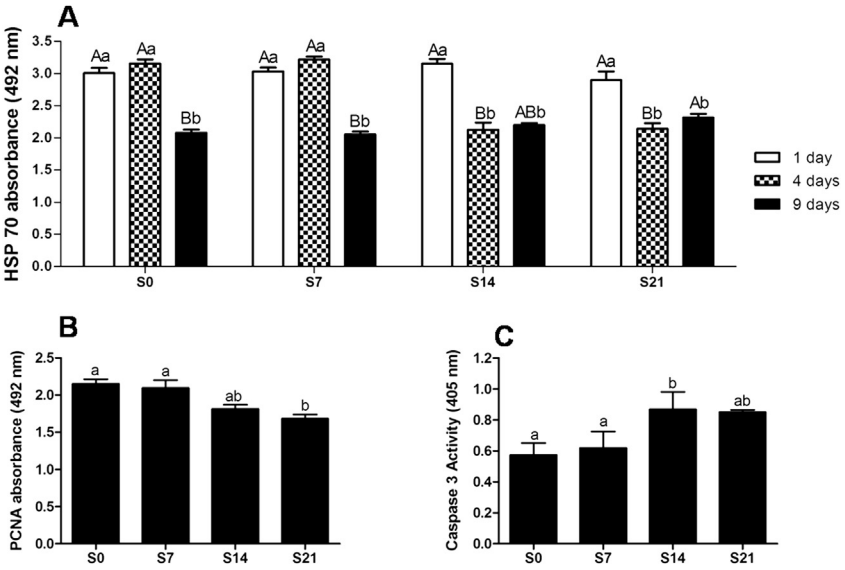

Fig. 3. Testicular levels of (A) HSP70, (B) PCNA and (C) caspase-3 by ELISA. Values expressed as means ± standard error. Different letters indicate significant difference between treatments ($p < 0.05$). Capital letters indicate variation between times and lower case letters between treatments.

affect the proportion of germ cells in the spermatogonial and meiotic phases. However, we found that spermiogenic phase is affected by increasing of water salinity, and this alteration leads to a decrease in the spermatozoa count. In general, germ cell apoptosis is common in the spermatogonial phase; however, in some fish species, it is higher during spermiogenesis [14,37]. The absence of variation in the germ cell proportion in the initial stages of spermatogenesis in the different treatments can be related to 11-KT plasma concentrations, since androgens act on the induction of spermatogenesis, mainly on germ cell proliferation and differentiation [38,39]. In the Atlantic salmon, small variations in the proportion of spermatogenic cells in different treatments suggest that salinity only affects the timing of spermatogenesis [36].

The 11-KT androgen plays a central role in fish spermatogenesis [40,41] and its levels are directly related to the number of germ cells [42,43]. In the present study, the higher proportion (%) of Leydig cells found in S_{14} and S_{21} may explain the increase in 11-KT plasma levels. Although 11-KT plasma levels showed no significant difference between treatments, the trend of higher values in S_{14} and S_{21} indicates a possible compensatory response to keep spermatogenesis in hypertonic media. According to Rocha and Rocha [44], the sex steroid levels present small variations in species with asynchronous germ cell development and multiple spawning such as the Nile tilapia. In addition, hyperplasia of Leydig cells not always reflects an increase androgen production. Evidences of studies where rats were subjected to stressful conditions like pollutants and heat exposure have shown that, even though the increased number of Leydig cells, the enzymes required for the steroids biosynthesis could be downregulated and key organelles like endoplasmic reticulum could be damaged [45,46]. For the Atlantic salmon, exposure to seawater did not affect 11-KT levels during puberty, yet levels of this androgen increased when high salinity and short photoperiod were conjugated, maybe reflecting in higher levels of hormone luteinizing (LH) to complete spermatogenesis [36]. On the other hand, hyperosmotic stress may stimulate the release of arginine vasotocin, an antidiuretic hormone which acts directly on the release of testicular androgens [47,48].

In teleost fish, the germ cells are distributed exclusively in cysts, suggesting that each cyst acts as an independent spermatogenic unit [11]. Furthermore, the presence of germ cells connected by cytoplasmic bridges can explain the complete emptying of the spermatogenic cysts in specimens from S_{21} in this study. Likewise, lacunae (empty cysts) possibly resulting from germ cells loss were also observed in zebrafish testes exposed to environmental oestrogens and anti-androgens in combination [49]. Given the presence of intercellular bridges between germ cells that originate from a common spermatogonial progenitor, the removal of some cells manifests in whole germ clones in apoptosis [14,50,51]. Thus, pro-apoptotic stimuli as well as pro-spermatogenic signals should act on the whole cyst in teleost fish.

In fish, several environmental and physiological factors stimulate cells to rapidly synthesise heat shock proteins (HSPs), which regulate cell metabolism, growth, differentiation, and apoptosis [19,52]. Hence, the decline in HSP70 levels observed on the 4th and 9th days of treatment in S_{14} and S_{21} may justify the increase in the caspase-3 detected in this study. These results suggest that high salinities negatively affect HSP70 expression, consequently increasing testicular apoptosis. In fact, HSP70 prevents apoptosis by binding to Apaf-1, causing its inhibition and, consequently, the activation of caspase-9 and caspase-3 becomes infeasible [53]. Besides increasing apoptosis, HSP70 can also modulate transcriptional activity and stability of androgen receptor (AR) [54]. In prostate cancer cells, the inhibition of Hsp70 expression suppresses AR expression and overexpression of HSP70 increase the AR protein levels. Thus, HSP70 expression is essential for testicular development and spermatic production in Nile tilapia.

In addition to its role as an effector molecule of the apoptosis, caspase-3 may also be involved in the cytoplasmic reduction of the spermatids during spermiogenesis [55]. In *Drosophila*, the apoptotic machinery is used to remove unnecessary cytoplasmic content in the formation of spermatozoa [56]. In addition, a study conducted on rats also showed that spermatids present histological and molecular evidence of apoptosis, such as vacuolization and the presence of apoptosis regulatory proteins [57]. In the present study, the presence of empty cysts at the highest salinities is a clear evidence of cell death by apoptosis occurring mainly in fish from S_{21}. This loss of germ cells through apoptosis during spermatogenesis plays a critical role in determining spermatogenic efficiency [58].

Proliferating cell nuclear antigen (PCNA) has been used in immunohistochemical studies as a biomarker of cell proliferation in

a variety of normal and pathological tissues, including testes [14,21,59]. In the present study, high salinities resulted in lower testicular PCNA levels and the immunolocalisation occurred in spermatogonia. PCNA is commonly detected during the spermatogonial phase in several fish species [14,18,60] and it also supports DNA repair in the meiotic phase [61]. As detected in this study, an increase in apoptosis and decrease in germ cell proliferation was found in males of *Prochilodus argenteus* exposed to low water temperature in a site impacted by a hydroelectric dam, when compared to a non-impacted site [15,62]. Thus, an imbalance between cell proliferation and apoptosis interfered negatively in the testicular homeostasis in Nile tilapia of this study.

In conclusion, despite of some molecular and histopathological alterations found in specimens of S_{14} and S_{21}, the results of this study indicate that salinity up to 21 g L^{-1} have few effects on spermatogenesis of Nile tilapia. The down-regulation of HSP70 in the 4th day treatment can be responsible by an imbalance in testicular homeostasis, with an impact on cell proliferation and apoptosis, resulting in reduced spermatogenic efficiency in specimens of S_{14} and S_{21}. Because it is a euryhaline fish with great plasticity, the molecular alterations found were subtle even in the higher salinities, which allow this species to colonize several environments. Thus, this study indicates the need to monitor salinity it as a tool for fish biodiversity conservation in continental waters.

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Authors' contributions

ABCV contributed to experimental procedures and manuscript preparation. AAW contributed to experimental procedures and statistical analysis. YMR and NB contributed in manuscript preparation. RKL contributed to experimental procedures. ER contributed to experimental design and manuscript preparation.

Declaration of interest

The authors declare no competing or financial interests.

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Appendix A. Supplementary data

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